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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61F 2/02	A1	(11) International Publication Number: WO 91/16009
		(43) International Publication Date: 31 October 1991 (31.10.91)

(21) International Application Number: PCT/US91/02662

(22) International Filing Date: 16 April 1991 (16.04.91)

(30) Priority data:
513,284 17 April 1990 (17.04.90) US
650,561 1 February 1991 (01.02.91) US

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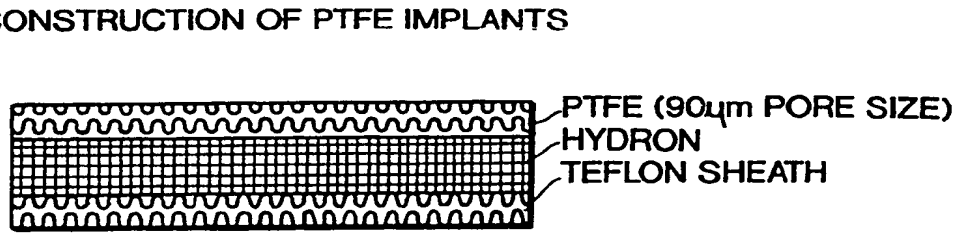
(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent), SU.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: COATING PROSTHETIC SURFACES WITH MAMMALIAN CELLS



(57) Abstract

A method of coating a prosthetic surface with mammalian cells is disclosed involving placing against the prosthetic surface a composition comprising a substance causing directed growth of endothelial cells and contacting the prosthetic surface, having the composition placed against it, with tissue or a physiological fluid, the tissue or physiological fluid containing mammalian cells, under conditions suitable for the mammalian cells to coat the prosthetic surface. The invention also includes a prosthesis with a surface coated as above with mammalian cells, as well as a method of treating mammals by implanting the above-described prosthesis.

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COATING PROSTHETIC SURFACES WITH MAMMALIAN CELLS

FIELD OF THE INVENTION

This application is a continuation-in-part of U.S. Patent Application Serial No. 513,284 filed April 17, 1990. This invention relates to methods of coating a prosthetic surface with mammalian cells. In particular, the invention relates to methods for treating the prosthetic surface with a substance causing directed growth of endothelial cells, and contacting the treated prosthetic surface with a tissue or physiological fluid containing mammalian cells, such that the mammalian cells coat the prosthetic surface.

BACKGROUND OF THE INVENTION

In the prior art there are many attempts, in the field of vascular grafts, to attain optimum levels of thrombogenicity and patency. Optimum thrombogenicity is attained when clot formation in the vascular graft has been minimized. Patency refers to vascular grafts in which blood flow is unhindered either by solid masses formed from blood constituents (clots) or by tissue infiltration and hyperplasia from cellular and tissue constituents associated with the graft. Experimental attempts to improve thrombogenicity and patency of vascular

grafts have included alteration of surface changes of biomaterials, treatment of biomaterial surfaces with anticoagulant substances, application of plasma polymerized monomers to the surfaces of biomaterials, and transplantation of endothelial cells onto biomaterial surfaces.

Endothelial cells are cells that line the cavities of the heart and the lumens (cavities or channels) within blood vessels, including capillaries. The presence of an endothelial lining produces a non-thrombogenic surface, which might be an ultimate requirement of a biomedically compatible and therefore biomedically useful vascular graft. Recent studies have shown the importance of chemotactic and mitogenic agents in regulating the activities of endothelial cells. Chemotaxis is the directed locomotion of cells along a chemical gradient. Mitogenic agents (mitogens) are those agents that cause cell populations to proliferate, i.e. agents that cause cell division. Chemotactic and/or mitogenic agents may be produced by, among other sources, blood platelets, macrophages, neural tissues, fibroblasts, and endothelial cells.

The prior art is illustrated by Zetter and Antoniades, V. Supramolecular Structure 11:361-370 (1979), which is incorporated herein in its entirety by reference thereto. This reference shows isolation of a particular mitogenic factor, platelet-derived growth factor (PDGF), from human blood platelets. Pure PDGF was shown to be mitogenic for human umbilical vein endothelial cells only when assayed in medium containing whole blood serum and thrombin.

Greisler and Kim, Seminars in Thrombosis and Hemostasis 15:206-214 (1989), which reference is incorporated herein in its entirety by reference thereto, hypothesize that macrophages in the vicinity

of copolymeric bioresorbable vascular grafts phagocytize (engulf) portions of the graft implants and are thereby influenced to release mitogens that facilitate endothelialization of the implant. Greisler and Kim observed macrophages in the vicinity of such implants in vivo. In vitro (in cell culture), they observed that phagocytosis of the copolymer substrate by macrophages correlated with the presence in the culture medium of substances mitogenic for mouse capillary lung endothelial cells. However, when vascular prostheses were coated with endothelial cell growth factor (ECGF), no enhancement of endothelialization was observed in vivo in several different experiments.

Rupnick et al., J. Vascular Surgery 9:788-795 (1989), incorporated herein in its entirety by reference thereto, shows the relative efficacy of two different methods for formation of endothelial cell (EC) monolayers on vascular grafts in vitro. The authors concluded that plating of EC cells onto preformed clots placed on the graft material was more effective than seeding of EC cells onto the graft surface in the presence of platelet-rich plasma, although loss of loosely adherent cells in response to shear stress was observed regardless. ECGF and heparin were not required for formation of EC monolayers on top of preformed clots and under the specific in vitro culture conditions reported in this reference.

Leseche et al., Surgery 105:36-45 (1989), incorporated herein in its entirety by reference thereto, shows the effect of endothelial cell growth supplement (ECGS) and heparin on in vitro proliferation of endothelial cells from stripped varicose veins. Such endothelial cells, when frozen-thawed, retained the ability to form in vitro monolayers on polytetrafluorethylene (PTFE) grafts, when the medium

was supplemented with ECGS and heparin. Ability of these monolayers to withstand physiologic arterial shear stress was not investigated.

The above studies demonstrate the need for methods to reliably coat prosthetic surfaces with clinically useful cell layers capable of maintaining patency and of withstanding shear stresses and other physiological challenges. In particular it would be useful to exploit chemotactic substances, allowing not only growth, but directed growth, of cells to the surface of prosthetic grafts and implants. It is an object of the present invention to employ a substance causing directed growth of endothelial cells to coat prosthetic surfaces with mammalian cells.

SUMMARY OF THE INVENTION

The present invention provides a method of coating a prosthetic surface with mammalian cells, comprising placing against the prosthetic surface a composition comprising a substance that causes directed growth of endothelial cells. The method further comprises contacting the prosthetic surface, having the composition placed against it, with tissue or a physiologic fluid containing mammalian cells, under suitable conditions for the mammalian cells to coat the prosthetic surface. The substance may be an isolate of blood or a synthetic product having the bioactivity of an isolate of blood. The substance further may be chemotactic for endothelial cells. The endothelial cells may be capillary endothelial cells.

Where the substance is an isolate of blood, the isolate may comprise a platelet releasate product where the platelet releasate product constitutes the materials released by platelets in the platelet release reaction or a fraction of the materials

released by platelets in the platelet release reaction. The platelets may be activated by an activator such as thrombin, adenosine diphosphate, collagen, cell disruption such as freeze thaw or other known means of activation. The composition which contains the product of platelet activation may be substantially free of blood or plasma contaminants or of platelet ghosts or other material found in platelets but not released by platelets upon activation.

Alternatively, the composition may contain an isolate of blood comprising a macrophage releasate product where the macrophage releasate product constitutes the materials released by macrophages in the macrophage release reaction or a fraction of the materials released by macrophages in the macrophage release reaction.

Where the substance is a synthetic product, the substance may be produced by polypeptide synthesis, recombinant DNA techniques, or other well-known methods of synthesis.

The prosthetic surface may be porous, and may have pore sizes averaging in the range of about 0.1 to about 250 micrometers ("um" or "u"). A porous prosthetic surface may be composed of any known biomedically useful polymer or silicon-based material, which is illustrated by dacron, polytetrafluorethylene, polymers of lactide-glycolide, polyglactin, polydioxanone, polyurethane or silicon-based material.

The prosthetic surface may be nonporous and may be composed of any biomedically compatible nonporous material, which is illustrated by stainless steel, titanium, cobalt chrome alloys, silicon-based material, or any of the polymers listed above.

The prosthetic surface may be the surface of tissue, an organ, or an aggregate of animal cells

or genetically altered cells, where the prosthetic member is a piece of tissue, an organ for transplantation or an aggregate of animal cells or genetically altered cells for cell implantation. Alternatively, the prosthetic surface may be a container, such as a polymer, having inside such tissue, organ or cell implant. Cell implants might be useful for treating a variety of conditions including diabetes, hemophilia, Parkinson's Disease, immune or blood disorders, etc. The coating of such surfaces, whether it be the tissue, organ, cell implant itself or a container having the same inside, may be useful to prevent macrophage or other degradation of such surface and also useful to induce vascularization of such tissue, organ or cell implant.

The prosthetic member may be a conduit where the prosthetic surface is the lumenal surface or the outer surface of the conduit. The conduit may be a duct implant, as illustrated by urinary ducts, kidney tubules, lymphatic ducts, bile ducts, pancreatic ducts, indwelling catheter, shunts, drains, or other known biomedical or anatomical ducts. Alternatively, the conduit may be a vascular implant.

The composition containing the substance causing directed growth of endothelial cells may be placed against the prosthetic surface by coating the prosthetic surface with the composition. Alternatively, the composition may be placed against the prosthetic surface by impregnating or coating a mandril with the composition and by placing the impregnated or coated mandril against the prosthetic surface.

The prosthetic surface of the conduit may be contacted with tissue containing mammalian cells, which are illustrated by endothelial cells, smooth muscle cells, fibroblasts, or other cells

known to be useful for maintaining the patency and/or clinical usefulness of duct or vascular implants. The prosthetic surface of the conduit may be contacted with such tissue by implanting the conduit into the retroperitoneal tissue or connective tissue of a mammal. If the conduit is a vascular implant, the conduit may, in addition, be contacted with such tissue by grafting the conduit to replace or bypass one or more blood vessels.

Alternatively, the prosthetic surface of the conduit may be contacted with physiological fluid containing mammalian cells such as endothelial cells, either in vivo (within the body) or in vitro. The lumenal surface of the conduit may be contacted with the physiological fluid by immersing the conduit in the physiological fluid in vitro or by passing the fluid through the lumen in vitro.

The prosthetic surface may be the surface of a stent, artificial joint, urological implant, patch, web, or other known forms of prostheses.

The invention further comprises a coated prosthetic surface prepared as described above, and a method of treatment of mammals comprising implanting the prosthetic device.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a longitudinal sectional view of the relative arrangements of the Teflon sheath, Hydron solution, and PTFE tube in an assembled PTFE implant.

Figure 2 shows a plot of 280 nm absorbance versus time for the chromatographic separation run in which fraction 1 was isolated.

Figure 3 shows ability of PDGF-BB and PDGF-AB to induce chemotaxis in rabbit wound capillary endothelial cells.

Figure 4 shows binding of ^{125}I-PDGF-BB

to receptors of rabbit wound capillary endothelial cells.

Figure 5 shows the effect of PDGF-BB on rabbit wound capillary endothelial cells proliferation.

DESCRIPTION OF THE PREFERRED EMBODIMENT

In accordance with the present invention, a method of coating a prosthetic surface with mammalian cells employing a composition comprising a substance causing directed growth of endothelial cells is provided.

The substance may be isolated from blood in the form of the product of platelet activation. Preferably, the substance may be the materials released by platelets ("platelet releasate product") in the platelet release reaction, as described in Knighton et al., Annals of Surgery 196:379-388 (1982), incorporated herein in its entirety by reference thereto, and as described below in Example 1. Alternatively, the product of platelet activation may comprise a fraction of the materials obtained upon platelet activation.

The biological activities of platelet releasate product were measured in a corneal implant assay designed to reveal ability to cause directed growth of endothelial cells (Example 2), and an endothelial cell chemotaxis assay (Example 3). Platelet releasate product, whether the materials released by platelets in the platelet releasate reaction or a fraction thereof, displayed positive responses in these assays. Isolation of a platelet releasate product comprising a fraction of the materials released by platelets in the platelet release reaction (hereinafter "fraction 1") is shown in Example 4. Platelet releasate product was negative in a rabbit wound endothelial cell mitogenic assay, adapted with minor modifications from the assay described

in Takahara et. al., Cell 49:415-422 (1987). Proliferation of endothelial cells in response to platelet releasate product was measured in this assay by monitoring cell numbers. On the other hand, platelet releasate product is mitogenic for mouse 3T3 fibroblasts. The fibroblast mitogenic assay (FMA) is described in Example 5.

In the preferred embodiment, a solution of 30% w/v polymer, available as Hydron® polymer, type NCC, cell culture grade (available commercially from HydroMed Sciences, New Brunswick, NJ 08901), 1.0% v/v polyethyleneglycol in 70% ethanol was prepared (hereinafter Hydron solution). Fraction 1 was diluted 1:3 with distilled water (dH_2O) and mixed 1:1 with the Hydron solution. For placebos the buffer used to isolate fraction 1, that is, 20 mM Tris, pH 7.4, 400 mM NaCl (without platelet releasate product), was diluted 1:3 with dH_2O and then mixed 1:1 with the Hydron solution.

An equal number of 16-gauge catheter sheaths were coated with each of the above-described fraction 1- or placebo-containing Hydron solutions. The catheter sheaths, also referred to herein as "mandrils", are manufactured of Teflon and are available from Jelco, Tampa, FL 33607. The mandrils were coated by dipping in the appropriate fraction 1- or placebo-containing Hydron solution, dried two hours under vacuum, recoated with the same solution, and dried under vacuum overnight. The sheaths were then placed within 2.5 cm long, 2.0 mm internal (lumenal) diamter polytetrafluoroethylene (PTFE) tubes (commercially available from Impra, Inc., Tempe AZ, or other suppliers). The PTFE tubes have a pore size of approximately 90 u. After placement of the mandrils within the PTFE tubes, the ends of the tubes were heat sealed. One-half of each

fraction 1 group and one-half of each placebo group had placed in the lumen of the mandril a piece of wire to give the implant assembly a slight bend. The relative arrangements of the Teflon mandril (sheath), the Hydron solution, and the PTFE tube are shown in longitudinal section in Fig. 1.

A total of 36 male inbred Lewis rats, about 250 gr of body weight, 8 to 10 weeks old, were housed in cages kept at 24°C and about 50% relative humidity. Water and Purina rat chow were continuously available. The room was illuminated for 12 hours in alternation with 12 hours of darkness (LD 12:12). The light was set automatically to come on at 06:00. The animal care complied with the "Principles of Laboratory Animal Care" (formulated by the National Society for Medical Research) and the "Guide for the care and use of laboratory animals" (NIH publication No. 80-23, revised 1985).

The animals were divided into three groups: A) a control group for the surgical technique (12 rats), B) a control group, placebo-treated for the study of the <u>in vivo</u> ingrowth of the endothelial cells (12 rats) and C) the study group given fraction 1 (12 rats).

Control Group for the Surgical Technique

6 animals were killed after the harvesting of the inferior vena cava utilized in the recipient animals as a by-pass procedure along the abdominal aorta. The inferior vena cava was chosen to match the available PTFE grafts in length and diameter. From each donor animal, the vena cava was harvested by carefully dividing between ligatures all the branches from the origin of the iliac vessels to the confluence of the left renal vein.

In the recipient 6 rats, the infrarenal abdominal aorta was prepared for end-to-side by-pass

anastomoses with the harvested veins. Each anastomosis was performed with a standard running technique, under dissecting scopy (magnification 6x) and with 10/0 monofilament sutures. Before the revascularization of each graft, the aorta within the anastomoses was occluded by transverse small metallic clips.

PTFE Graft Implantation

Twenty-four rats, 12 for each treatment group, were anesthetized by a single intraperitoneal injection of sodium pentobarbital (60 mg/kg bw) and the abdominal cavity entered through a midline incision. Each animal received 2 tubes of the same treatment group: 1 bent tube placed, after a longitudinal incision of the posterior layer of the peritoneum, into the retroperitoneal tissue lying on the infrarenal abdominal aorta, and 1 straight tube placed into the retroperitoneal tissue below the right kidney.

The animals were evenly assigned to 6 implantation schedules (4 animals per schedule, 2 animals per treatment group) in order to implant the grafts at 6 different timepoints, 4 hours apart, at 04:00, 08:00, 12:00, 16:00, 20:00, and 00:00 clock hours. According to the lighting regimen in the room, the groups of animals were, therefore, implanted at 02, 06, 10, 14, 18, 22 hours after light on (HALO).

The tubes were left in place for 3 weeks. The straight ones were then taken out for analysis, while the bent ones, after removal of the inside Teflon sheath and wire, were <u>in situ</u> revascularized end-to-side to the underlying abdominal aorta. The technique was the same as that utilized for the vein in the control group. Before revascularization, the aorta within the anastomoses was occluded by small metallic clips.

Conventional Histology

After ex vivo formalin fixation of 5mm length of each tube, standard H and E staining was performed on cross-sectional slices. A semiquantitative grading of each slide was then assigned as follow:

Full and complete ingrowth of endothelial cells	4
Full but incomplete ingrowth of endothelial cells	3
Isolated areas of endothelial cell ingrowth	2
Almost no endothelial cell ingrowth	1

Postoperative Evaluations

In the 14th postoperative day, each animal was examined for the patency of the by-pass. Under anesthesia, the abdominal cavity was entered and the flow velocity recorded with a 8 MHz doppler probe (BV102R Vascular Flow Detector, available from Sonicaid, Inc., Fredericksburg, VA 22404) at the level of the by-pass and on the distal abdominal aorta. The occluded by-passes were then removed from analysis and the animals killed. The functioning grafts were left in place for 3 more months in order to evaluate the long term patency rate. At that point, each animal was killed and the grafts removed for the scheduled analysis. Results of these experiments are shown in Table I, below.

TABLE I

GROUPS	14 DAYS		100 DAYS	
	THROMBOSED	PATENT	THROMBOSED	PATENT
VEIN GRAFTS	0	6 (100%)	0	5 (100%)**
PTFE GRAFTS (Placebo)	4 (37%)*	7 (63%)	2 (33%)***	4 (67%)

PTFE GRAFTS* (Fraction 1)	1 (9%)	10 (91%)	2 (20%)	8 (80%)

*1 technical failure excluded from analysis

**anesthesia death

***1 animal excluded from analysis due to tissue abnormality around the graft

As can be seen from the above data, PTFE grafts that had been treated with fraction 1-containing Hydron solution gave higher patency rates compared to the placebo group. Histology of the fraction 1-treated grafts revealed the presence of endothelial cells, smooth muscle cells, and fibroblasts within the tissue lining the lumenal surface of the PTFE tubes.

Example 1

Platelet Release Reaction

60 ml whole blood was aeseptically obtained from a source in 6 ml of acid citrate dextrose anti-coagulant (hereinafter ACD), or 1 ml ACD per 10 ml of whole blood. The blood was mixed well with ACD by inverting and rolling the syringe. Anti-coagulated blood samples were kept on ice until used in further processing.

The anti-coagulated blood was transferred to two sterile, siliconized 50 ml conical-bottom centrifuge tubes, evenly splitting the sample between tubes. The tubes were then centrifuged at 135 x g for 20 minutes at about 4°C. Upon completion of the centrifugation cycle, the rotor was allowed to coast to a stop. No braking was applied. The uppermost layer of the centrifuged sample, platelet-rich plasma (hereinafter PRP), was carefully transferred with a sterile pipette to another sterile, siliconized centrifuge tube. Drawing only 4-5 ml at a time minimized

losses due to red blood cell contamination of the PRP. A platelet count of the PRP was then conducted using methods well known in the art.

The PRP was centrifuged at 750 x g for 10 minutes at about 4°C. The supernatant was discarded, being careful not to dislodge the platelet pellet. Using a sterile pipette, the pellet was resuspended by aspirating and expelling into buffer containing 0.05 M HEPES (N-2-hydroxyethyl piperazine-n-2 ethane sulfonic acid), 0.03 M dextrose, 0.004 M KCl, 0.1 M NaCl, pH adjusted to approximately 6.5 at 28°C (hereinafter platelet buffer) to an approximate concentration of 10^9 platelets per ml of suspension.

The resulting platelet suspension was then activated with purified thrombin. Preferably, about 1 unit of thrombin per ml of platelet suspension was added to the platelet suspension and mixed. The platelets and thrombin were allowed to incubate at room temperature for about 10 minutes. After incubation, the resulting platelet aggregate was broken up by aspirating and expelling the suspension with a sterile pipette.

Alternatively, the platelet suspension may be activated with other activators that cause the platelets to release their contents. Other activators include collagen, preferably 6-100 ug of monomer collagen per ml of buffer containing 10% platelets, ADP, preferably 2-10 u molar in said buffer, epinephrine, preferably 25-450 u molar in said buffer, and arachidonic acid, preferably 35-50 u molar in said buffer.

As a further alternative embodiment, PRP can be activated with thrombin or otherwise before centrifugation.

In the preferred embodiment, the resulting

supernatant was centrifuged at 950 x g for about 5 minutes at about 4°C, thereby removing the released platelet ghosts and any fibrin contained in the suspension. The pellet formed by such centrifugation was discarded after the supernatant was extracted.

After removal of the platelet ghosts and fibrin, the remaining supernatant constitutes platelet releasate in platelet buffer, herein designated platelet releasate product. The extract is frozen in 4 ml aliquots for storage or immediately used.

Platelet releasate product also may be prepared from platelets obtained from a blood bank or other source. Pheresis platelet concentrate may be obtained from a blood bank and immediately processed. One unit of platelets will yield approximately 200 mls of PRP.

The concentrate may be processed to produce the activated platelet suspension in the same manner as the anti-coagulated patient blood sample is processed above, except that the first platelet pellet obtained from PRP is centrifuged three additional times at 750 x g for 10 minutes at about 4°C, resuspending the platelet pellet in platelet buffer after each centrifugation. After the final centrifugation, the platelet pellet is resuspended in platelet buffer to an approximate concentration of 10^9 platelets/ml.

The platelet suspension is activated as described above and centrifuged at 950 x g for 10 minutes at about 4°C. The supernatant is extracted and centrifuged at 10,000 x g for 15 minutes at about 4°C to remove residual platelets and any fibrin. The pellet is discarded after the supernatant is extracted. The supernatant which is the platelet releasate product is frozen in 4 ml aliquots for storage or immediately used.

As a further alternative to production

from blood bank platelets, PRP produced from banked platelets can be directly activated before centrigugation.

Example 2

Rabbit Implant Corneal Assay

2-4 polymer pellets were made for each sample to be tested for angiogenic activity. A solution of 10% w/v polymer, available as Hydron® polymer, type NCC, cell culture grade, (Available commercially from Interferon Sciences, New Brunswick, NJ 08901), 1% v/v polyethyleneglycol in 70% v/v ethanol should be prepared (hereinafter polymer solution). Polymer solution is mixed 1:1 v/v with test sample. A piece of plastic autoclave bag is taped onto a flat surface, making sure it is taut. The surface is then wiped off with an alcohol prep, and allowed to dry. 20 ul of the 1:1 mixture is dropped onto the plastic. The polymer pellets are then dried under vacuum for approximately 2 hours, or until dry.

The corneal implant assay is conducted on a 4-6 lb. New Zealand White Rabbit. Anesthetic is prepared by mixing 1:1 v/v Ketamine hydrochloride 100mg/ml, commercially available as Ketaset® from Veterinary Products, Bristol Laboratories, Syracuse, NY 13201, and acepromazine maleate 10mg/ml, commercially available as Promace® from Aveco Co., Inc., Fort Dodge, IA 50501, in the same syringe. 4-5cc is used for each rabbit. Anesthetic is injected into the gluteus maximum or gastrocnemeus using a 23 gauge needle, gently rubbing the area after injection. The rabbit is properly anesthetized when it cannot resist being rolled onto its back, usually in 10-15 minutes.

The rabbit is placed on a sterile drape. 3-5 drops of proparacine hydrochloride 0.5%, commercially available as Ophthetic® from Allergan Pharmaceuticals,

Inc., Irvine, CA 92713, are put in each eye to numb the area. The anesthetic solution is used as needed throughout the procedure whenever the eye becomes dry.

The eye is brought out of the socket using petite point tissue forceps. The forceps are slowly worked towards the inner corner of the eye and a bit of tissue is clamped to ensure the eye remains in this position while working, taking care not to clamp the optic nerve.

A scapel, Beaver eye blade No. 5210, commercially available from Beaver Surgical Products, Waltham, MA 02154, is gently drawn across the apex of the cornea, making an incision approximately 3.0 mm long. It is possible to puncture the cornea which will cause the aqueous humor to seep through. If this should occur, the animal must be sacrificed.

With a Elschnig cyclodialysis spatula, 1mm wide, 10mm long commercially available from V. Mueller, Chicago, IL 60648, product #OP-2040, a canal is gently made through the cornea towards the capillary bed, stopping approximately 2 mm from the capillary bed. A "pocket" is made for the polymer pellet by moving the tip of a probe side to side, taking care not to move the probe forward as the pellet should not be closer than 1 mm to the capillary bed. A polymer pellet is lifted off the plastic using forceps and placed on the eye at the point of incision. With a spatula, the pellet is pushed through the canal and into the pocket. Several drops of anesthetic solution is used to lubricate the area and make insertion of the pellet easier. The pellet must be concentrated in the pocket. Trapped air is pushed out from the pocket by drawing a spatula along the canal on the outside of the cornea.

The forceps are then unfastened. The eyelids

are gently pulled up and out manually and the eye resumes its normal position. Three drops of anti-bacterial solution, commercially available as Neosporin® Opthalmic Solution from Burroughs Wellcome Co., Research Triangle Park, NC 27709, are put into each eye to minimize the possibility of infection.

One rabbit is used for each sample to be tested (i.e., 2 pellets of same sample per rabbit, one in each eye).

Eyes are checked on days 3, 5 and 7 for any direct growth of capillaries towards the pellet and graded according to the method of Gimbrone et al., J. Natl. Cancer Inst. 52:413-427 (1974), and Banda et al., U.S. Patent No. 4,503,038, both of which are incorporated in their entirety by reference. Pictures of eyes are taken on day 7 to record capillary growth.

Example 3

Endothelial Cell Chemotaxis Assay

PREPARATION OF CELLS

1. Grow Rabbit Wound Capillary Endothelial Cells (RWCE) to 60-85% "confluent" on 3-4 75 cm^2 flasks (Falcon #3824 or #3047, Collaborative Research, Inc., Bedford, MA). Rabbit Wound Capillary Endothelial Cells may be prepared as described in copending application Serial No. 337,284 filed April 13, 1989, the disclosure of which is incorporated herein by reference.

2. Approximately 20-24 hours before chemotaxis, remove the media and rinse flasks twice (2X) with HBSS* (Ca/Mg free, 6 ml/flask).

3. Remove last HBSS wash and add 12-15 ml (be consistent) of 0.2% lactalbumin in Media 199* to each flask. (This provides minimal nutrients and reduces serum induced stimulation, so the cells are ready to respond to the attractants.) Record time of media change on flask.

4. The following day, prepare the following:

   a) 50-100 ml 0.2% lactalbumin in M100 (LA-M199)

   b) 20-30 ml (5 ml/75cm^2 flask) Enzyme Cocktail No. 2 (EC2-1X)* by diluting 1 ml EC2(10X) in 9 ml HBSS.

5. Remove 0.2% LA-M199 and rinse flask with 6-10 ml HBSS*. Immediately add 5 ml EC2(1X)* and incubate for exactly 14 minutes at R.T.

6. Pool EC2 from flasks into 50 ml polypropylene tube(s) containing at least 2 ml/flask 0.2% LA-M199, to help deactivate the enzymes. Immediately add 5 ml 0.2% LA-M199 to each flask.

7. Gently scrape off cells from bottom with a sterile cell scraper (American Scientific Products Cat. #T4206-1).

8. Add cells/media to the EC2 pool. For a final rinse, add 10 ml 0.2% LA-M199 to one flask. Transfer rinse wash from flask to flask, then pool with cells.

9. If final volume exceeds 40 ml, divide cells into two tubes for centrifugation. Centrifuge cells at 1400 rpm (approx. 450g on Mistral 3000 centrifuge) for 10 minutes at R.T. or 1,000 rpm (approx. 250 g on Beckman GPR).

10. Discard supernatant by quickly pouring off. Resuspend pellet(s) in 8 ml total 0.2% LA-M199 (pool if divided) and transfer to a 15ml centrifuge tube. Wash tubes with an additional 2 ml media and add to resuspended cells. Centrifuge at 1400 rpm for 10 minutes at R.T.

11. Resuspend cells in 2-5 ml 0.2% LA-M199 (adjust volume to size of pellet and estimated cell yield: to avoid recentrifugation) for counting.

12. Count cells:

   a) Add 30 ul cell suspension to 30 ul

Trypan Blue.

b) Load both sides of heamocytometer.

c) Under 10X mag., count cells in eight 1mm squares. Record number of viable (blue) cells. Do not count cells of abnormal size or shape.

d) Multiply viable cell count by 2.5×10^3 to = cells per ml.

13. Adjust cell concentration to 0.75×10^6 cells/ml LA-M199 (i.e., 33,750 cells per/well in 45 ul). Approximately 2.25 ml/chamber is needed.

a) Example: There are 3 ml of 1.5×10 cells per ml. The final adjusted voluem should be:

$$\frac{(3 \text{ ml}) (1.5 \times 10^6 \text{ cells/ml}}{(0.75 \times 10^6 \text{ cells/ml}} = 5.95 \text{ ml}$$

Therefore, add 2.95 ml 0.2% LA-M199 to cells to obtain a final concentration of 0.75×10^6. In short, prepare 45 wells worth of cells/chamber at a concentration of 33,750 cells/45 ul 0.2% LA-M199/well.

*Suggested Volumes

Flask size (cm^2)	0.2% LA-M199 feed media (ml)	HBSS wash (ml)	EC2(1X) (ml)
25	5	5	3
75	15	6-10	5
150	30	10-15	10

PREPARATION OF FILTERS

1. Prepare 20 ml of 1 ug Fibronectin (Sigma #F4759)/1 ml HBSS (FN/HBSS) from frozen stock. Use polypropylene tips and tubes only for its preparation. (Example: Frozen stock = 1 ng/ml dH_2O-HBSS. Therefore dilute 200 ul stock in 19^2.8 ml HBSS). *Store on ice until use.

2. Use Nuclepore polypropylene filters (8.0 um pores, PVPF, from Neuro Probe

Inc., 301-229-8598), one per chamber.

3. Cut the upper left-hand corner off the shiny side of the* filter to provide filter orientation. Use tweezers to handle the filter, (never fingers) and only to the ends.

4. Place 3-4 ml FN/HBSS in the center of a sterile petri dish. Lay filter on top of the FN/HBSS, shiny side down, allowing to spread beneath the filter; do not allow any FN/HBSS on the top of the filter.

5. Cover petri dish and allow to stand at R.T. for 30 minutes.

6. Carefully pour off FN/HBSS (by tilting majority to one side slowly, allowing filter to stick to plate, then pouring off completely), lift up fitler, place 3-4 ml fresh FN/HBSS in center and repeat procedure on other side of filter (dull side down).

7. Coating is now complete and filters can be used.

*Note: a) For consistency, use filter immediately, by timing the filling of the bottom chamber to coincide with the completion of coating the second side of the filter.

b) When preparing two filters, it is recommended to stagger their coating times by 15 minutes to allow for the filling of one chamber before having to fill the second, to alleviate having to rush to fill both at the same time.

PREPARATION OF CHAMBERS

1. Remove Neuro Probe 48 well chemctaxis chambers from distilled water (dH_2O) storage bath. Rinse thoroughly with clean dH_2O. Dry top piece and gasket with tissue, and blow dry bottom piece with clean nitrogen gas.

2. Prepare test samples to load bottom of chamber. Each well holds approximately 26.0 - 26.5 ul) solution.

3. Add (approximately 26.1 - 26.5 ul) samples to the wells in the lower chamber.

*Note: a) To produce a desired "positive meniscus", slight "topping off" with fluid may be necessary. This helps to counteract drying which occurs while filling the rest of the chamber. Avoid creating bubbles.

b) Use a positive displacement pipette for best consistency. Since the first and last columns (A,L) of four wells on either chamber end are not utilized for chemotaxis, fill them with HBSS. Consequently, these same rows in the upper chamber are also filled with HBSS and not cells.

4. When the filters are ready, pour off FN/HBSS (as previously described). Carefully lift the filter off the petri dish; do not let either side scrape against the edge of the dish. By lifting filter slowly, the residual FN/HBSS on the filter is minimized. DO NOT drop or carelessly touch the filter at this point.

5. While holding both ends of the filter with forceps, lower the center of the filter onto the center of the chamber, then evenly cover bottom wells. The filter should be shiny side up!

*Note: Always orient chamber/filter consistently; i.e., always keep chamber trademark and cut corner on filter in the upper left-hand corner.

6. Only if necessary, adjust the filter slightly to properly align.

7. Lay the gasket just above the filter, but not touching.

8. Place the upper half of the chamber on top of gasket and push down both together. Hold them down tightly while securing the retaining screws.

9. Once secure, pick up chamber and look through wells for any bubbles that may have formed underneath filter; record these, as they can interfere with chemotaxis.

10. Add 45 ul HBSS to the four wells on both ends (A,L).

11. Next add 45 ul of cell suspension (i.e., 0.75×10^6 cells per ml) to the remaining wells.

*Note: Add cells with pipette tip at an angle to prevent trapping air in the bottom. If a bubble forms, carefully withdraw liquid and refill. After filling, all wells should look uniform. If not, suspect trapped air and redo.

12. Place the chamber in a glass or polypropylene tray, add a water soaked gauze square (increasing humidity and preventing evaporation), and loosely cover with aluminum foil.

13. Incubate for 4 hours at 37°C, 5% CO_2.

REMOVAL AND WIPING OF FILTER

1. Etch a glass slide at one end with date and chamber number. Clean well with alcohol prep and dry.

2. Remove retaining nuts while holding down top plate.

3. Orient chamber with trademark in upper left-hand corner, over a paper towel.

4. Invert entire chamber (along horizontal axis) onto paper towel.

5. Push down on the four corners f the top plate so it stays parallel to the bottom plate as it drops. The filter should be stuck to the gasket.

6. Remove the bottom plate and immerse immediately in Tergazyme solution (1/4 teaspoon Tergazyme/1000 ml dH_2O).

7. The "migrated cells" are now facing up. DO NOT disturb this face of the filter from here on.

8. Catch the very right-hand edge of the filter with forceps, lift edge to loosen, then pull filter slightly to the right so the end just hangs over the edge.

9. Clamp this end with the plastic clip and lift the filter off the gasket. Quickly apply the second plastic clip to the other end. Place top chamber piece immediately in Tergazyme or distilled H_2O.

10. Keeping the cell side up (ALWAYS), wet the non-migrated side in PBS. Do not let PBS wet the "migrated cells" side.

11. Holding the filter taut, draw the non-migrated side against the wiper blade (from one end to the next in one direction only).

12. Repeat this procedure 4-5 times. Minimize time between wetting and wiping to prevent non-migrated cells from drying/sticking and causing incomplete removal. ALWAYS dry wiper blade before each successive wipe.

13. Place filter on appropriate etched slide, with cut corner on same end as etching, but on opposite side. Allow to dry overnight.

14. Rinse off chamber pieces sitting in Tergazyme with dH_2O and store in fresh dH_2O, covered, until chambers are to be cleaned.

STAINING OF FILTER

Set up the Densitometer (LKB) so the filter can be read immediately after staining.

1. Place small black clip on the end of the dried filter/slide with the cut

corner.

2. Stain in LeukoStat (Fisher brand) by dipping in each of three solutions, in order, 5 times for 5 seconds each time. Dab off excess stain on a paper towel or gauze between solutions.

3. Allow filter to sit in third stain for 30 seconds extra after the 5 dips.

4. Rinse filter in dH_2O (use two changes of dH_2O). Dab excess dH_2O off.

5. Place another clean glass slide (unmarked) directly over the filter, and press together carefully yet firmly, forcing out most of the air bubbles.

6. Read on the Densitometer.

DENSITOMETRY READING OF STAINED FILTER

1. Allow Densitometer (LKB) to warm up for 10-20 minutes.

2. Place stained, wet slide on reading table and orient to proper coordinates as follows:

Column	X Position	"Track"
B	113.6	1
C	119.6	2
D	127.0	3
E	132.6	4
F	138.6	5
G	146.6	6
H	152.6	7
I	158.0	8
J	166.0	9
K	171.6	10

Additional densitometer settings:

a) Smoothing: 3
b) x-width: 4
c) y-start: 19
d) y-stop: 43

3. Line up slide. Check column positions.

4. Clip slide down without moving it.

5. Check "Y" coordinates on various rows.

6. Send ruler "home". "Ecs".

7. Close lid.

8. "Enter" (on computer), then "6" (or "Run") on Densitometer.

9. Calculate area of peaks from Densitometer using LKB's "GSXL" program.

Alternatively, stain filter in Diff-Quik (Baxter Scientific Products, Minneapolis, MN) and repeat steps 1-4 in above staining protocol and count cells in microscope using 10 x 10 after allowing to dry following step 4. This alternative procedure will not require use of densitometer.

CLEANING CHAMBERS

The following procedure is used to remove residual proteins, etc., from the chemotaxis chambers and gaskets. (Source: Terri Superdock, 118:61, 2/21/89).

1. Rinse dirty gaskets and chambers well with deionized water. Place chambers with corresponding gaskets in a 1 liter plastic beaker (2 sets/beaker).

2. Heat 0.75% Tergazyme solution (7.5 gm Tergazyme/1 liter dH_2O; 500-750 ml/2 chambers) to 50°C. DO NOT EXCEED 50°C.

3. Cover chambers and gaskets with 50°C Tergazyme.

4. Place beaker(s) in a 50°C waterbath. Cover bath and incubate for 2 hours.

*For gasket cleaning see steps 10 and 11.

5. Remove chambers only and rinse well with dH_2O. Place chambers in a 1 liter plastic beaker (1000 ml); 2-3 chambers/beaker.

6. Cover chambers with R.T. 1 M NaOH (600-700 ml/beaker). Cover beaker

with tin foil.

7. Incubate beaker(s) in a 50°C covered waterbath for 30 minutes.

8. Rinse chambers very well with dH_2O. Place chambers and a large stir bar in deep plastic tub. Strategically orient chambers (tops and bottoms) so as not to interfere with the spinning stir bar. Put tub on a magnetic stirrer near a sink.

9. Fill tub with dH_2O, letting it run continuously for 2 hours. Make sure the water is circulating adequately, and a syphoning system is placed in the tub leading to the sink to prevent overflow.

10. Place gaskets into one beaker with 0.75% Tergazyme and sonicate for 30 minutes.

11. Rinse well with dH_2O and place gaskets in 1 liter dH_2O. Sonicate for 2 hours, changing the water every 30 minutes.

12. Assemble chambers and gaskets (lightly tighten with screws) and place in flat pan (polypropylene) filled with fresh dH_2O. Cover with aluminum foil, and change water once a week.

13. Rinse chambers and gaskets well with fresh dH_2O before use.

Example 4

Preparation of Fraction 1

A platelet pheresis preparation was obtained from the University of Minnesota blood bank and processed substantially as described in Example 1. The centrifuged platelets were resuspended in platelet buffer at a concentration of 5×10^9/ml and released with Thrombin (Thrombinar,® NDC 0053-7100-01, Armour Pharmaceutical Co., Kankakee, Illinois 60901) at a ratio of 5 units of Thrombin per ml. Following the platelet release reaction, the solution was dialyzed against two changes

of chromatographic column run buffer (20 mM Tris, pH 7.4, 50 mM NaCl), four hours total, 4°C.

The chromatographic column was a Mono-Q anion exchange column, 1.0 cm (ID) X 5.0 cm (available from Pharmacia Co., Piscataway, NJ, serial number 7393041). The protein concentration of the releasate solution was estimated from the 280 nm U.V. absorbance, and 5-10 mg of total protein was loaded on the column.

A step gradient was prepared by appropriate combinations of the following buffers: A = 20 mM Tris (pH 7.4); B = A + 1.0M NaCl (pH 7.4). The column was run as follows:

	Time Interval	NaCl
1.	0-10 min.	70 mM
2.	10-20 min.	140 mM
3.	20-35 min.	190 mM
4.	35-45 min.	400 mM
5.	45-50 min.	400 mM-1000 mM
6.	50-55 min.	50 mM

The flow rate for time intervals 1, 2, 3, 4 and 6 was 1.0 ml/min. The flow rate for time interval 5 was 0.5 ml/min. The chromatographic system was a Beckman System Gold (modified), with individual components as follows: a) Pump: Beckman Model 126, b) Detector: Beckman Model 167, variable wavelength, U.V./visible, c) Controlling computer: IBM Model PS 2/50, d) Printer: Epson Model 100, e) Fraction collector: Gilson Model 201.

The absorbance at 280 nM U.V. of the fractions collected from the column is shown in Fig. 2. Fractions collected in the time interval of 32-39 min. were tested in the chemotaxis assay described in Example 3, above. Results were as follows:

Fraction	cells/field	mean+ S.D.
Positive Control*	129, 90, 118, 159	124 + 29
Negative Control**	31, 16, 43, 104	49 + 39
32-33 min.	19, 18, 120, 104	65 + 54
33-34 min.	42, 24, 176, 69	78 + 68
34-35 min.	92, 51, 94, 96	83 + 22
35-36 min.	380, 260, 245, 355	310 + 67
36-37 min.	128, 144, 78, 120	118 + 28
37-38 min.	45, 41, 59, 78	56 + 17
38-39 min.	83, 78, 196, 55	91 + 39

*Positive Control: 1:10 dilution of total platelet releasate reaction

**Negative Control: 0.2% lactalbumin hydrolysate

From the above data, it can be seen that the fraction collected at 35-36 min. was most active in the chemotaxis assay. This fraction, designated "fraction 1" and shown in Fig. 2, was used for the PTFE studies described above.

Example 5

Fibroblast Mitogenic Assay

1. Determine the number of microtiter plates necessary for FMA samples to be tested. (One plate will accommodate 24 quadruplicate or 32 triplicate samples "including" necessary controls).

2. Prepare approximately 20 ml per plate of Delbucco's Modified Eagle Medium (DMEM) containing 10% newborn calf serum (NCS). Prepare an additional 40 ml DMEM/10% NCS (used for preparing the cells).

3. Thaw out appropriate number of tubes of 3T3 (A31) fibroblasts, which are stored frozen in liquid nitrogen, in a 37°C water bath. (Yield per tube

will vary with frozen batch. Approximately 2,000,000 viable cells per microtiter plate will be needed.)

4. Aseptically transfer cells to sterile 50 ml culture tube (12 ml and 15 ml can also serve this purpose, just cut back on the liquid) containing 20 ml (5-10 ml) DMEM/10% NCS. "Resuspend well" and centrifuge at 450xg (1400rpm in Mistral 3000i with shielded swing bucket rotors) for 10 minutes at R.T. Decant supernatant, resuspend cellular pellet in 10 ml DMEM/10% NCS, transferring it to a sterile 12 ml culture tube. Repeat centrifugation.

5. Resuspend cellular pellet in approximately 2-5 ml DMEM/10% NCS. Perform cell count.

6. Dilute the cells in DMEM/10% NCS to obtain a concentration of 200,000 cells per ml. (For each plate 10-11 mls are needed).

7. Add 100ul per well to 96 well microtiter dish using an 8 or 12 multi-channel pipettor and sterile boat reservoir. Be sure to draw suspension in and out of the pipettor at least once per row to maintain cell-suspension adequately).

8. Add 100ul DMEM/10% NCS to each well (for a combined total of 200 ul liquid per well).

9. Label plates with cell line and date of plate preparation. Incubate plates at 37°C, 5% Co_2 for 3 days or until fibroblasts are confluent.

MEDIA CHANGE/DAY 3

Three days after initiating the microtiter plates, the media needs to be changed to 0.8% NCS/DMEM to continue.

1. Examine the plates under the microscope to determine if the fibroblasts have grown to confluency. (There should be no gaps between cells). If cells are confluent, continue. If cells

are not confluent, they can be grown for one extra day, or discarded.

2. Prepare 16 ml/plate 0.8% NCS/DMEM.

3. Open and place a sterile barrier sheet under hood. One at a time, take plates to sink and carefully flick all the liquid out of the plate in one clean, gentle sweep. Replace cover immediately.

4. Return quickly to sterile hood and gently blot opened plate on the sterile barrier to remove excess liquid.

5. Immediately and gently add 150 ul 0.8% NCS/DMEM per well using an 8 or 12 channel pipettor. Care must be taken to avoid disturbing the confluent cells as much as possible. Repeat steps 3 through 5 with next plate(s).

6. Incubate plates at 37°C, 5% CO_2 for 6 hours.

7. Retain excess 0.8% NCS for making dilutions.

STIMULATE CELLS/6 HRS. POST MEDIA CHANGE

Six hours after changing the media from 10% to 0.8% HI-CS, the cells are ready to be stimulated.

1. Fill out the template for each microtiter plate outlining the location of each control and sample to be tested.

2. Beginning in the upper left corner, the first 3 or 4 wells receive 50 ul of 0.8% NCS/DMEM only. (This serves as the plate background control).

3. The next 3 or 4 wells (working horizontally) receive both 20 ul undiluted NCS and 30 ul 0.8% NCS per well. (Final dilution therefore = 10% NCS).

4. The next 3 or 4 wells receive 50 ul of Platelet Buffer control (10 ml Platelet Buffer + 50 ul thrombin).

5. Add 50 ul of the test/control samples.

6. Incubate the plates 37°C at 5% CO_2 for 18 hours. (Consistency in timing is important).

RADIOACTIVE LABELING

Eighteen hours after stimulation with test and control samples, the FMA microtiter plates are labeled with radioactive thymidine to demonstrate mitogenic activity.

1. Line work surface area with disposable paper liner to contain any accidental spills while working with the radionuclides. Protective gloves should be worn.

2. Prepare a 10 uCi [3H]-thymidine/ml DMEM solution as follows: Sterile transfer 0.5 cc [3H]-thymidine (NEN cat no. NET-027, 6.7 C mmol, 1mC /ml) to 49.5 ml DMEM (a 1/100 dilution).

3. Add 50 ul of [3H]-thymidine/DMEM solution to each well. Store leftover radioactive solution in the refrigerator for next time.

4. Properly discard pipette tips, gloves, dispensing container holding the aliquot of labeled media, and paper liner in the radioactive waste.

5. Label plates as radioactive and incubate in a tray to contain any spills at 37°C, 5% CO_2 for 6 hours.

HARVESTING

1. Carefully aspirate off radioactive culture media using a NUNC immuno wash. Be sure to use "raise pins" provided, to prevent contact of aspiration prongs with the cells.

2. Wash cells by adding 200 ul PBS with multi-channel pipettor. Aspirate with NUNC immuno wash.

3. Add 200 ml 10% by volume trichloroacetic acid (TCA), place on ice for 5 minutes and remove TCA and repeat.

4. Add 200 ml 0.5M NaOH and incubate at room temperature.

SCINTILLATION PREPARATION

1. Add 100 ml of sample from 96 well dish and add 4 ml scintillation cocktail (Beckman Ready-Safe) to each vial.

2. Vials are capped tightly and shaken back and forth vigorously a few times to expose filter completely to the cocktail and disclodge potential air bubbles.

COUNTING

1. Place vials into the Beckman LS1701 green racks in order from left to right or in Beckman 2800.

2. If B-LS1701, place "program rack" into counter first, consisting of an empty green rack with ONE vial in the 18th position, telling the machine to use Program No. 1.

3. Program No. 1 is programmed as follows:

B-LS1701	B-2800
- Replicates: 3	4
- Count time: 2 minutes	1
- H#: No	No
- Sample Repat: 1	1
- Data calc: CPM	CPM
- SCR: Yes	No
- RCM: Yes	No
- Vial size: Mini	-
- Count Blank: No	-

4. Place remaining racks into counter working "back to front" on the right side first, then "front to back" on the left side. Always end with the RED stop rack.

5. Push both "RESET" buttons at the same time.

6. When RESET is complete, and the printer has been checked for enough paper, press the START button and replace the cover.

7. Monitor the initial print-out to confirm accuracy of program being used.

Units of 1/ED-50 represents the dilution of the platelet releasate sample which results in a 50% stimulation of mitrogenic activity in fibroblast 3T3 cells. For example, if a 0.25 to 1:4 dilution of the sample gave 50% stimulation, the 1/ED-50 would be 4 units. Similarly, a 1:8 dilution would give an 1/ED-50 of 8 units.

DESCRIPTION OF ALTERNATIVE EMBODIMENTS

In addition to materials derived from the platelet release reaction, the substance causing directed growth may be derived from other means of platelet activation such as platelet disruption involving freeze/thaw or heat treatment of platelets or may be derived from any isolate of blood, or any other material, which causes directed growth of endothelial cells. With procedures well known in the art for chemical synthesis of polypeptides, for production of polypeptides through recombinant DNA technology, and other procedures, the substance may be a synthetic product having the bioactivity of an isolate of blood.

An example of an isolate of blood and recombinant DNA produced peptide is platelet derived growth factor. Platelet derived growth factor ("PDGF") is a cationic glycoprotein of about 32,000 molecular weight sourced from platelets by release reaction or freeze/thaw or heat treatment. PDGF may also be isolated from multiple tissues and cultured cells, including macrophages, smooth muscle cells, tumor cells and endothelial cells. The predominant form of PDGF secreted by platelets is a heterodimer consisting of an A chain and a B chain, and a less dominant form (about 20%) being the homodimer of the B chain. The following examples will show that the homodimer of B chain ("PDGF-BB") is chemotactic and nonmitogenic

for wound capillary endothelial cells, while the heterodimer of A chain and B chain ("PDGF-AB") is marginally chemotactic for wound capillary endothelial cells.

Example 6

Recombinant human PDGF-BB ("rhPDGF-BB") was obtained commercially (Upstate Biotechnology, Inc., Lake Placid, NY 12946, Catalog #01-105: rhPDGF-BB expressed in S. cerevisiae) and recombinant human PDGF-AB ("rhPDGF-AB") was also obtained commercially from the same source (Catalog #01-109: rhPDGF-AB expressed in E. coli). rhPDGF-BB and rhPDGF-AB obtained in lyophilized form was rehydrated to 5.0 ug/ml in PBS (containing 1.0 mg/ml HSA). The rehydrated dimers are stored at -20° C until use. Rehydrated rhPDGF-BB and rhPDGF-AB were assayed according to the endothelial cell chemotaxis assay of Example 3. Figure 3 shows the concentration in ng/ml of rhPDGF-BB and rhPDGF-AB respectively, the rehydrated dimers being further diluted in M199 containing 0.2% by volume lactalbumin. According to the results shown in Figure 3, rhPDGF-BB induces chemotaxis of rabbit wound capillary endothelial cells (RWCEC), while rhPDGF-AB marginally induces such chemotaxis.

Example 7

Rabbit wound capillary endothelial cells (RWCEC) were cultured, passaged, and enumerated according to the above protocol for culture of RWCEC. Following isolation of RWCEC from culture and enumeration, the RWCEC are diluted in Media 199 (M199) (#320-1150, Gibco Laboratories, Grand Island, NY) containing 10% by volume rabbit serum (FRS) and 5% by volume fetal bovine serum (FBS) (#240-6000, Gibco Laboratories, Grand Island, NY) to a concentration of $5X10^4$/ml. These culture conditions are described in the RWCEC culture protocol. One ml of the cell suspension

is pipetted into each well of a 24-well dish (Falcon 3047, Becton Dickinson Labware, Oxnard, CA) which has been precoated with a 1/100 dilution of Matrigel (#40234, Collaborative Research, Inc., Bedford, MA) in calcium/magnesium-free Hanks Balanced Salt Solution #310-4170, Gibco Laboratories, Grand Island, NY). The cells are cultured 3-4 days to reach confluence.

In preparation for the binding assay, the cells are washed, 1 ml per well, with phosphate-buffered saline (#450-1300, Gibco Laboratories, Grand Island, NY) containing 1.0 mg/ml human serum albumin (NDC 0944-0490-01, Baxter Healthcare Corp., Glendale, CA) (PBS-HSA) cooled to 4^{o} C and all subsequent operations are performed with precooled reagents and at 4^{o}C. The 24-well dish is placed onto a rocker platform for 30 minutes. After 30 minutes, the cells are washed again with PBS-HSA and incubated an additional 30 minutes. Following the wash step, 200 ul of PBS-HSA is added to each well along with 25 ul of the appropriate dilution of ^{125}I-PDGF-BB (#IM.213, Amersham Corp., Arlington Heights, IL) to achieve the desired final concentration (0.5, 1.0, 3.0, 5.0, and 10 ng/ml is common for the Scatchard plots, 3 ng/ml is standard for the competition studies).

For each concentration of ^{125}I-PDGF-BB used, two wells receive 25 ul of unlabeled PDGF-BB to achieve a concentration that is 100 fold in excess of the amount of ^{125}I-PDGF-BB while the other two wells receive 25 ul of PBS-HSA to bring the final volume of all wells to 250 ul. Concentrations were prepared with rehydrated dimers in PBS (containing 1.0 mg/ml HSA). For competition studies where only one concentration of ^{125} I-PDGF-BB is used all wells receive that concentration of ^{125} I-PDGF-BB, two wells will then also receive a 100 fold excess of PDGF-BB for determination of nonspecific binding,

while the other wells will receive the material being tested for competitive activity (in duplicate) such as PDGF-AA (#01-109, Upstate Biotechnologies, Inc., Lake Placid, NY) or PDGF-AB (#01-110, Upstate Biotechnologies, Inc., Lake Placid, NY). The dishes are then incubated for 1 hour at 4^{o} C on the rocker platform with constant rocking. Following the incubation each well is washed 3 times with 1.0 ml of cold PBS-HSA and the cell associated radioactivity is extracted by the addition of 250 ul of 0.5% by volume Triton X-100 (#X-100, Sigma Chemical Co., St. Louis, MO) in PBS. The plates are allowed to sit overnight at room temperature and the radioactivity of each sample is determined in a gamma counter (Gamma 5500, Beckman Instruments, Inc., Irvine, CA). The dishes are thoroughly mixed and 100 ul of each well is counted and the total multiplied by 2.5 to get the total amount of radioactivity per well.

The specific binding is determined by subtracting the mean of the duplicates of the samples containing ^{125}I-PDGF-BB + a 100 fold excess of PDGF-BB from the mean of the duplicates of the samples containing only ^{125}I-PDGF-BB for each concentration of ^{125}I-PDGF-BB tested. If performing Scatchard plots, the number of cells per well is determined by counting four replicate wells in the same dish. The cells are removed with trypsin-EDTA (per the RWCEC proliferation assay) and enumerated in a hemocytometer as described in the RWCEC culture procedure. Using these values the Kd (dissociation constant) of the receptor and the receptor number per cell can be calculated by the method of Scatchard (Scatchard, G., The Attraction of Proteins For Small Molecules and Ions, Ann. NY Acad. Sci. 51:660-672 (1949)). Figure 4 shows the presence of a high affinity receptor for PDGF-BB with a Kd of 0.1-0.3 nM and a receptor number of

3-4 X 10^4/cell.

Example 8

Rabbit wound capillary endothelial cells (RWCEC) were cultured, passaged, and enumerated according to the above protocol for culture of RWCEC. Following isolation of RWCEC from culture and enumeration, the RWCEC are diluted in Media 199 (M199) (#320-1150, Gibco Laboratories, Grand Island, NY) containing 10% by volume rabbit serum (FRS) and 5% by volume fetal bovine serum (FBS) (#240-6000, Gibco Laboratories, Grand Island, NY) to a concentration of 5X10^3/ml. These culture conditions are described in the RWCEC culture protocol. One ml of the cell suspension is pipetted into each well of a 24-well dish (Falcon 3047, Becton Dickinson Labware, Oxnard, CA) which has been precoated with a 1/100 dilution of Matrigel (#40234, Collaborative Research, Inc., Bedford, MA) in calcium/magnesium-free Hanks Balanced Salt Solution #310-4170, Gibco Laboratories, Grand Island, NY). The cells are cultured at 37 degrees C in a 5% CO_2 atmosphere. The next day (Day 1) the media in three wells is replaced with 1 ml of M199 containing 2.5% by volume FBS and three wells receive 1 ml of M199 containing 10% by volume FBS. The remaining wells receive 1 ml of the test materials diluted to the appropriate concentration in M199/2.5% by volume FBS (each test material is tested in triplicate). The dishes are then returned to the incubator. On Day 3 the media in all of the wells is changed, with the material in each well being replaced with like, freshly prepared material. The dishes are then returned to the incubator. On Day 4 the media in each well is removed, each well is rinsed one time with 1 ml of phosphate-buffered saline (PBS) (#450-1300, Gibco Laboratories, Grand Island, NY) and 1 ml of 0.05% by volume trypsin/0.53 mM ethylenediaminetetraacetic

acid (Trypsin/EDTA) (#610-5300, Gibco Laboratories, Grand Island, NY) is added to each well and the plate incubated at 37 degrees C for 5 minutes to detach the cells. At the end of 5 minutes, the dish is checked in the inverted microscope to see that all cells are detached. The cells in each well are gently pipetted to insure homogeneity of the cell suspension. The 1 ml from each well is then added to 9 mls of Isoton (#375-220, Curtin Matheson Scientific, Minneapolis, MN), gently mixed, and the number of cells per well determined in an electronic cell counter (Coulter, Curtin Matheson Scientific, Minneapolis, MN) with the following settings: lower threshold-0.8, upper threshold-98.9, attenuation-32, and present gain-8.

Figure 5 shows that rhPDGF-BB is not mitogenic as measured by the above assay for rabbit wound capillary endothelial cells. Concentrations were prepared with rehydrated dimers in M199 containing 2.5% by volume FBS.

Example 9

rhPDGF-BB was bioassayed according to the corneal implant assay of Example 2. The following results show significant angiogenesis for rhPDGF-BB. Concentrations were prepared with rehydrated dimers in PBS (containing 1 mg/ml HSA).

Material	# Positive Corneas/Total	Average Score	Inflammation Day 2	Day 7
PBS/HSA	0/8	0	-	-
PDGF-BB				
10 ng/imp.	1/4	0.25	-	-
50 ng/imp.	4/6	1.5	-	
100 ng/imp.	5/6	2.5	-	-
250 ng/imp.	2/2	2.5	+	-
500 ng/imp.	4/4	4	++	+

In a further embodiment, the substance may comprise materials released from macrophages or a fraction of the materials thereof. Macrophages undergo a "macrophage release reaction" in the sense that, under some conditions (for example, stimulation by endotoxin), macrophages release materials capable of causing growth of endothelial cells and blood vessels in vivo and in vitro, as disclosed in Thakral et. al., J. Surgical Research 26:430-436 (1979), which is herein incorporated in its entirety by reference thereto.

The prosthetic surface may, depending on the material and the desired use, exhibit pore sizes other than approximately 90 um. For example, pore sizes averaging in the range of about 0.1 um to about 250 um, as well as other pore sizes compatible with the materials used and the intended use, may be employed. In addition to PTFE, porous prosthetic surfaces may be composed of any known biomedically useful polymer, such as dacron, polymers of lactide-glycolide, polyglactin, polydioxanone, or polyurethane. Porous silicon-based materials may also be used for prosthetic surfaces. Other biomedically useful materials for porous prosthetic surfaces will be known to those skilled in the art.

Prosthetic surfaces also may be fashioned from any biomedically compatible (i.e., tolerated by the body without clinically unacceptable adverse effects) nonporous material, such as stainless steel, titanium, cobalt chrome alloys, silicon-based materials, and other known materials.

The prosthetic surface could be the lumenal surface or the outer surface of a conduit-type prosthetic member, depending on the intended use. The conduit prosthetic member could be a vascular

implant, as described in the preferred embodiment. Alternatively, the conduit prosthetic member could be a duct-type implant, as illustrated by urinary ducts, kidney tubules, lymphatic ducts, bile ducts, pancreatic ducts, indwelling catheters, shunts, drains, or other known biomedical or anatomical ducts.

Alternatively, the prosthetic surface may be the surface of a stent, artificial joint, urological implant, patch, web, or other known forms of prostheses.

As described in the preferred embodiment, the composition may comprise the substance causing directed growth, plus a Hydron/polyethyleneglycol carrier. Other useful formulations of Hydron/polyethyleneglycol will be known to those skilled in the art, depending on the intended use. Useful carriers other than Hydron and/or polyethyleneglycol will also be known to those skilled in the art. The composition may be used to coat a mandril or other object, which is then placed against the prosthetic surface as described in the preferred embodiment. Alternatively, the mandril or other object may be impregnated with the composition (for example, by soaking an absorbent mandril in the composition), or the prosthetic surface itself may be coated with the composition.

The prosthetic surface in contact with the composition may be placed in an environment containing mammalian cells by implantation into retroperitoneal tissue of a rat, as described in the preferred embodiment. Alternatively, the prosthetic surface may be implanted into other appropriate mammalian tissues. In a further embodiment, the prosthetic surface may be contacted with physiological fluid such as various body fluids <u>in vivo</u> or such

as various formulations of cell culture media (in vitro contact) known to those skilled in the art. A conduit prosthetic member could be contacted with the physiological fluid by immersing the conduit in the fluid or by passing the fluid through the lumen of the conduit, in vivo or in vitro.

The mammalian cells within the tissue or physiological fluid may be any cells useful for maintaining the patency and/or clinical usefulness of duct or vascular implants. These could include, without limitation, endothelial cells, smooth muscle cells, and/or fibroblasts.

In the above embodiments, the prosthetic surface is brought into contact with the composition, then contacted with mammalian tissue or physiological fluid containing mammalian cells that will coat the prosthetic surface. It is at that point that the prosthetic member could be grafted or implanted to its final location as a functioning prosthetic device. In an alternative embodiment, the prosthetic member, with the prosthetic surface in contact with the composition, could be grafted or implanted directly to its final location, where mammalian cells inherently in the vicinity of the final location would be induced to coat the prosthetic surface.

From the foregoing, it will be apparent to those skilled in the art that various modifications in the above-described methods can be made without departing from the spirit and scope of the present invention. Accordingly, the present invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. Present embodiments and examples, therefore, are to be considered in all respects as illustrative and not restrictive, the scope of the present invention being indicated by the appended claims rather than

by the foregoing, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

CLAIMS

1. A method of coating a prosthetic surface with mammalian cells comprising:

placing against the prosthetic surface a composition comprising a substance where the substance causes directed growth of endothelial cells; and

contacting the prosthetic surface, having the composition placed against it, with tissue or a physiological fluid, said tissue or physiological fluid containing mammalian cells, under suitable conditions for said mammalian cells to coat the prosthetic surface.

2. The method of claim 1 wherein the substance is selected from the group consisting of an isolate of blood and a synthetic product having the bioactivity of an isolate of blood.

3. The method of claim 1 wherein the substance is chemotactic for endothelial cells.

4. The method of claim 3 wherein the endothelial cells are capillary endothelial cells.

5. The method of claim 2 wherein the substance is an isolate of blood.

6. The method of claim 5 wherein the isolate of blood comprises a product of platelet activation selected from the group consisting of the materials released by platelets in the platelet release reaction, a fraction of the materials released by platelets in the platelet release reaction, materials released by platelets upon activation by freeze/thaw or heat treatment of platelets and a fraction of materials released by platelets upon activation by freeze/thaw or heat treatment of platelets.

7. The method of claim 6 wherein the product of platelet activation is said fraction.

8. The method of claim 6 wherein the

platelets are activated by freeze/thaw or heat treatment.

9. The method of claim 6 wherein said platelets are activated by an activator selected from the group consisting of thrombin, adenosine diphosphate and collagen.

10. The method of claim 9 wherein the activator is thrombin.

11. The method of claim 9 wherein the composition is substantially free of (i) blood or plasma contaminants and (ii) platelet ghosts or other material found in platelets but not released by platelets in the release reaction.

12. The method of claim 5 wherein the isolate of blood comprises a macrophage releasate product selected from the group consisting of the materials released by macrophages in the macrophage release reaction and a fraction of the materials released by macrophages in the macrophage release reaction.

13. The method of claim 2 wherein the substance is a synthetic product having the bioactivity of an isolate of blood.

14. The method of claim 13 wherein the synthetic product is produced by polypeptide synthesis or recombinant DNA techniques.

15. The method of claim 1 wherein the prosthetic surface is porous.

16. The method of claim 15 wherein the prosthetic surface has pore sizes averaging in the range of about 0.1 u to about 250 u.

17. The method of claim 16 wherein the prosthetic surface is the surface of a biomedically

useful polymer or silicon-based material.

18. The method of claim 17 wherein the biomedically useful polymer or silicon-based material is selected from the group consisting of dacron, polytetrafluoroethylene, polymers of lactide-glycoclide, polyglactin, polydioxanone, polyurethane, and silicon-based material.

19. The method of claim 1 wherein the prosthetic surface is nonporous.

20. The method of claim 19 wherein the prosthetic surface is the surface of a biomedically useful polymer or silicon-based material.

21. The method of claim 20 wherein the biomedically useful polymer or silicon-based material is selected from the group consisting of dacron, polytetrafluoroethylene, polymers of lactide-glycoclide, polyglactin, polydioxanone, polyurethane, and silicon-based material.

22. The method of claim 19 wherein the prosthetic surface is the surface of a biomedically compatible nonporous material selected from the group consisting of stainless steel, titanium, cobalt chrome alloys and silicon-based material.

23. The method of claim 1 wherein said prosthetic surface is the lumenal surface of a conduit or the outside surface of a conduit.

24. The method of claim 23 wherein the prosthetic surface is the lumenal surface.

25. The method of claim 23 wherein the conduit is a duct implant.

26. The method of claim 25 wherein said duct is a member of the group consisting of urinary ducts, kidney tubules, lymphatic ducts, bile ducts,

pancreatic ducts, indwelling catheter, shunts and drains.

27. The method of claim 23 wherein the conduit is a vascular implant.

28. The method of claim 23 wherein the composition is placed against the prosthetic surface by coating the prosthetic surface with said composition.

29. The method of claim 23 wherein the composition is placed against the prosthetic surface by placing a mandril against said surface where said mandril has been impregnated with said composition.

30. The method of claim 23 wherein the composition is placed against the prosthetic surface by placing a mandril against said prosthetic surface where the surface of said mandril which opposes the prosthetic surface has been coated with the composition.

31. The method of claim 23 wherein said prosthetic surface of the conduit is contacted with said tissue and said mammalian cells comprise cells selected from the group consisting of (1) endothelial cells, (2) smooth muscle cells and (3) fibroblasts.

32. The method of claim 31 wherein said mammalian cells are endothelial cells.

33. The method of claim 31 wherein said prosthetic surface of said conduit is contacted with said tissue by implanting said conduit in the retroperitoneal tissue of a mammal.

34. The method of claim 31 wherein said prosthetic surface of said conduit is contacted with tissue by implanting said conduit into connective tissue of a mammal.

35. The method of claim 31 wherein said

conduit is a vascular implant and said lumenal surface of the vascular implant is contacted with tissue by grafting said vascular implant to replace or bypass one or more blood vessels.

36. The method of claim 23 wherein said prosthetic surface of said conduit is contacted with said physiological fluid and said mammalian cells are endothelial cells.

37. The method of claim 36 wherein said prosthetic surface of said conduit is contacted with said physiological fluid by immersing said conduit into said physiological fluid in vitro.

38. The method of claim 36 wherein said lumenal surface of said vascular implant is contacted with said physiological fluid by passing said physiological fluid through said lumen in vitro.

39. The method of claim 1 wherein said prosthetic surface is the surface of a stent or artificial joint or urological implants or patch or web.

40. The method of claim 1 wherein the prosthetic surface is the surface of animal tissue implant, animal organ implant or cell implant.

41. The method of claim 1 wherein the substance is PDGF-BB.

42. The method of claim 1 wherein the substance is the PDGF-B chain or a fragment of PDGF-B chain, said substance having the bioactivity of chemotaxis for capillary endothelial cells.

43. The method of claim 1 wherein the substance is PDGF-AB.

44. A prosthetic member comprising a coated prosthetic surface prepared according to

any of claims 1-43.

45. A method of treatment of mammals comprising implanting a prosthetic device of claim 44.

Fig. 1

CONSTRUCTION OF PTFE IMPLANTS

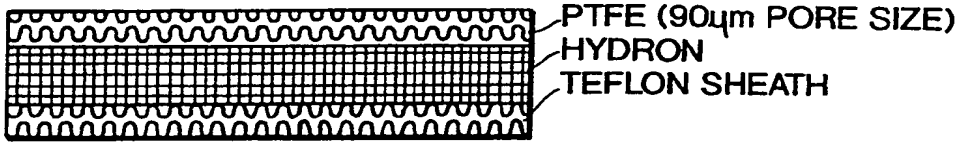


Fig. 3

ABILITY OF PDGF-BB AND PDGF-AB TO INDUCE RWCEC CHEMOTAXIS

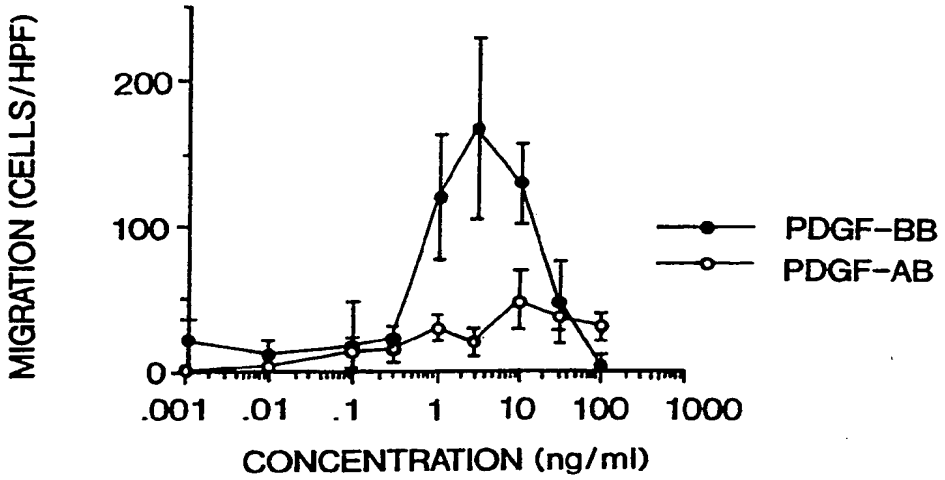
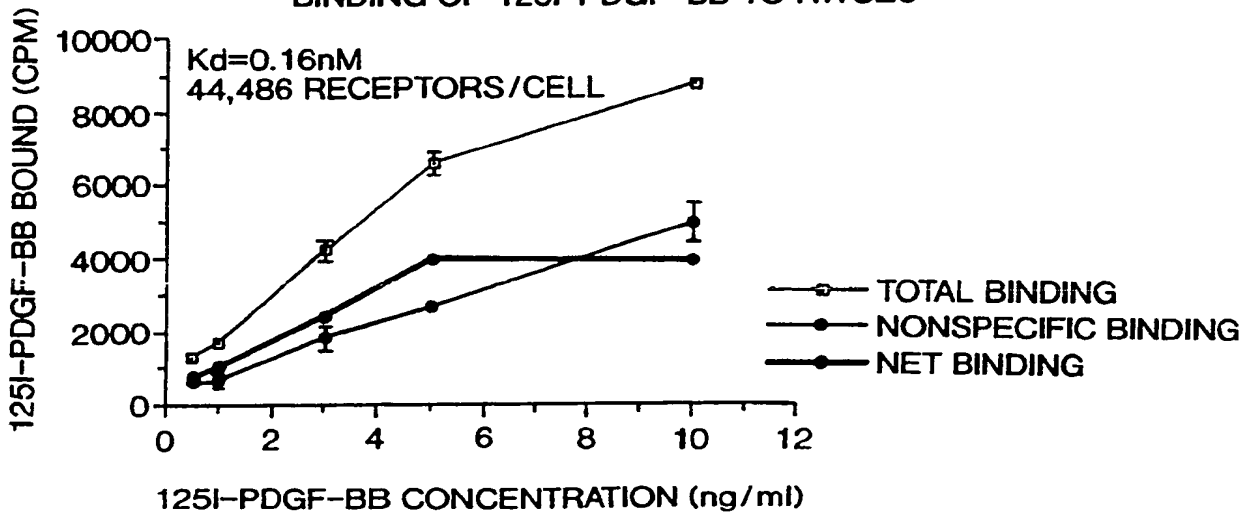


Fig. 4

BINDING OF 125I-PDGF-BB TO RWCEC



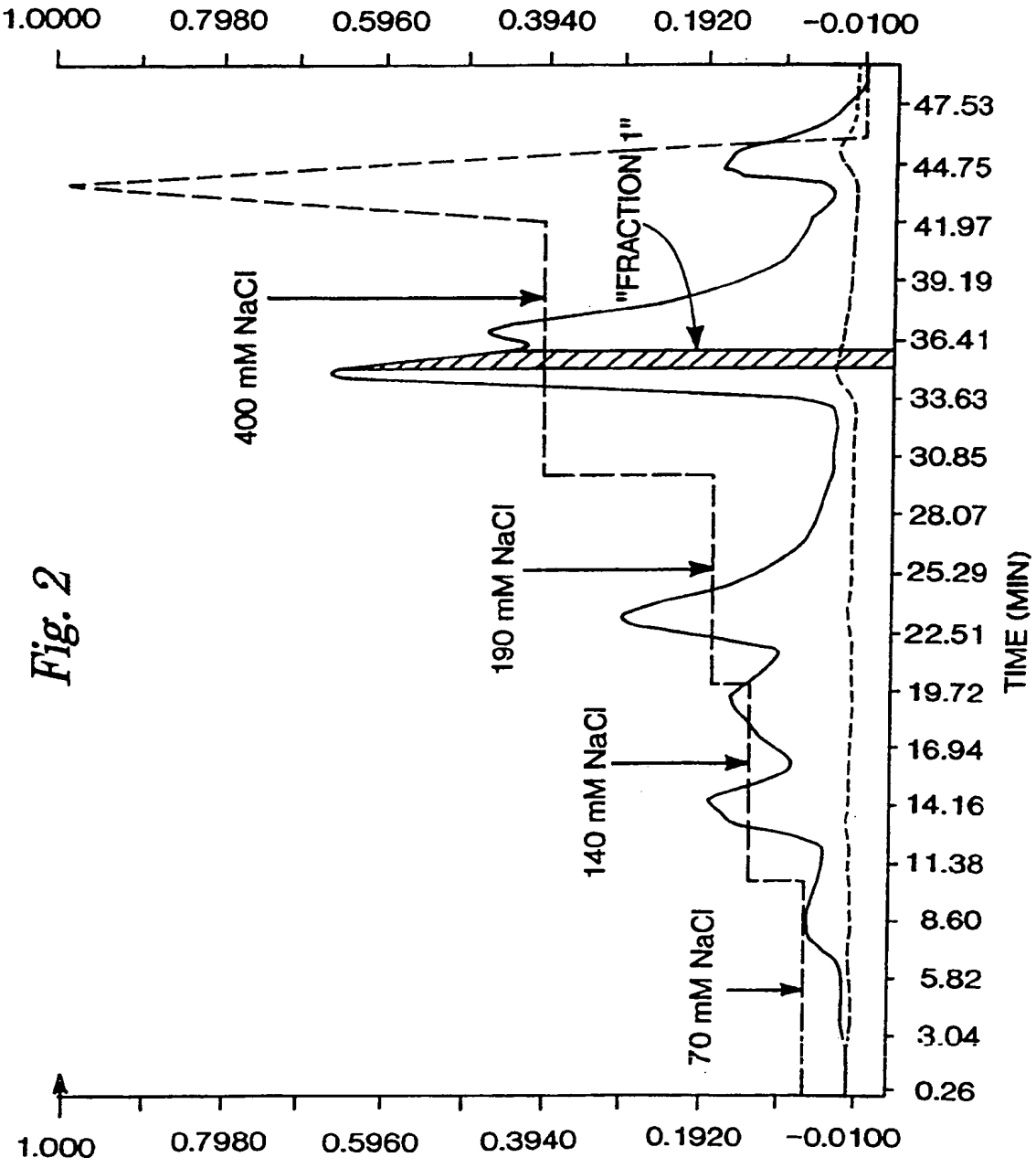
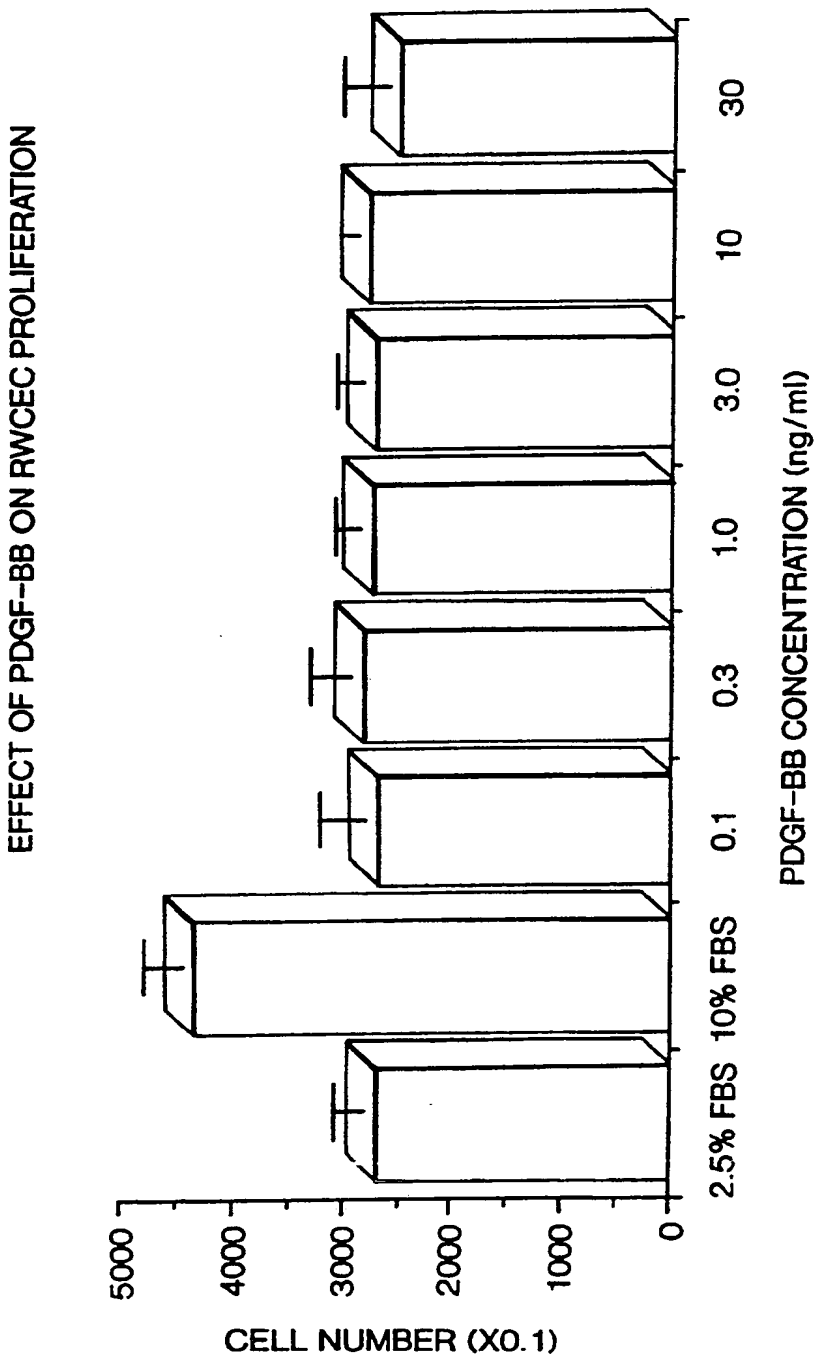

1.0000
0.7980
0.5960
0.3940
0.1920
-0.0100
47.53
44.75
41.97
39.19
36.41
33.63
30.85
28.07
25.29
22.51
19.72
16.94
14.16
11.38
8.60
5.82
3.04
0.26
TIME (MIN)
"FRACTION 1"
400 mM NaCl
190 mM NaCl
140 mM NaCl
70 mM NaCl
1.000
0.7980
0.5960
0.3940
0.1920
-0.0100
ABS @ 214 & 280 nm

Fig. 2

Fig. 5


EFFECT OF PDGF-BB ON RWCEC PROLIFERATION
CELL NUMBER (X0.1)
5000
4000
3000
2000
1000
0
2.5% FBS
10% FBS
0.1
0.3
1.0
3.0
10
30
PDGF-BB CONCENTRATION (ng/ml)

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/02662

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61F 2/02
US CL.: 623/1

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US	623/1,2,11,12,16,66

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	US, A, 4,546,500 (BELL) 15 October 1985 See columns 3 and 4.	1-12,15-18,23-28,36-41
Y	US, A, 4,589,881 (PIERSCHBADNER, et al.) 20 May 1986, see abstract.	13,14,19-22

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

20 JULY 1991

Date of Mailing of this International Search Report

03 SEP 1991

International Searching Authority

IPEA/US

Signature of Authorized Officer

DAVID J. ISABELLA

Form PCT/ISA/210 (second sheet) (Rev. 11-87)